CONCANAVALIN A INDUCED AUTOPHAGY SWITCHES TO APOPTOSIS

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CERTIFICATE

This is to certify that the thesis entitled "***Concanavalin A induced autophagy switches to apoptosis***" which is being submitted by Miss. Madhusmita Panda, Roll No. 412LS2049, for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bonafide research work, carried out by her under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

Dr. Sujit Ku. Bhutia

DECLARATION

I, **Miss. Madhusmita Panda having Roll No.412LS2049** hereby declare that the project report entitled '**Concanavalin A induced autophagy switches to apoptosis'** submitted by me, is an original work done and submitted by me in partial fulfilment for the Degree of Master of Science in Life Sciences to the NIT ,Rourkela. This is a project work done by me under the guidance of **Dr. Sujit Kumar Bhutia**, Assistant Professor, and NIT Rourkela. This thesis has not formed the basis for the award of any other Degree /Diploma/ Associateship/ fellowship or other similar title to any candidate in any university.

Date: **Madhusmita Panda**

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DEDICATED TO MY BELOVED PARENTS

CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

%: Percentage

°C: Degree Celsius

ConA: Concanavalin A

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl Sulfoxide

EDTA: Ethylene Diamine Tetra Acetate

FBS: Fetal Bovine Serum

gm.: Gram

Hrs: Hours

Kg: Kilogram

L: Liter

M: Molar

MEM: Minimum Essential Medium eagle

mg: Milligram

Min: Minute

mL: Milli Liter

mM: Milli molar

OD: Optical Density

PCD: Programmed Cell Death

CQ: Chloroquine

SDS PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

µg: Micro gram

µm: Micro meter

ABSTRACT

Concanavalin A (Con A) is a lectin or carbohydrate binding protein having broad range of significant biological functions such as anti-tumor, anti-viral, anti-inflammatory, anti-proliferative activities. The current study intends to focus on the role of Con A in cell death mechanisms autophagy and apoptosis in oral cancer cell line Hep-2. The acridine orange staining demonstrates that Con A is able to induce oral cancer cell autophgy. The ability of con A to induce apoptosis was studied using various staining procedure such as DAPI, Annexin V-FITC staining and Caspase-3/7 Glo assay. Additionally how chloroquine possesses the potential to inhibit the autophgic activity of con A has also been studied. As conclusion, the molecular mechanism of Con A induced cell death by autophagy followed to apoptosis in oral cancer has been well discussed, which will lead to opening of new perspectives for Con A as potential anti-cancer drugs in future oral cancer therapeutics.

Key words: Lectin, Con A, Cancer, Apoptosis, Autophagy

1. INTRODUCTION

Lectins are carbohydrate binding proteins which are non-immune in origin and specifically recognise the carbohydrate moieties. They bind with carbohydrates both reversibly and non-covalently. "Lectin" is derived from the Latin word "*Legere"*, means "to select" The ability of reorganisation and binding of specific carbohydrate structures is the biological function of lectins ([1]*Goldstein et al., 1980*). Not only lectins distinguish between different monosaccharide but they specifically bind to oligosaccharides also, by detecting suitable difference in complex carbohydrate structure. So far the function is concerned lectins can be differentiated according to their recognition to endogenous or exogenous ligands. Lectins recognizing endogenous ligands play a job in fertilization and development and sometimes their function also involves in cell-to-cell or cell-to-matrix interaction ([2]*Sharon, N. Lis,H.; 1989*). Another type of lectins which recognizing exogenous ligands are evolved for non-self discrimination and they might be soluble or surface bound. The main function of lectin in animals is cell adhesion to glycoprotein synthesis. Some lectins which are found on surface of liver cells in mammals for recognized galactose residues. In immune system their function is reorganisation of carbohydrates and found on pathogens or these are inaccessible on host cells. Lectins are used in various purposes specifically for medicine and medical research. Purified lectins are used for blood typing as clinical research. Lectins have been widely used as the molecular basis of protein recognizes carbohydrates. Another important type of the lectin family is the Legume lectins, which is mainly found in the seed of the legume plant. This legume lectin plays a potential role in cancer treatment ([3]*Gabius et al., 2011*).

1.1. Role of lectins in cancer treatments:

Plant lectins have a potent biological activity, which contain a unique group of proteins and glycoproteins generally found in foods like wheat, pea, tomato, corn, peanut, kidney bean, banana, soybean. Several lectins have anticancer properties which were found *in-vitro*, i*n-vivo* and in human case studies. They are used as therapeutic agents when binding to cancer cell membranes and their receptors. It causes cytotoxicity, apoptosis, and also inhibition of tumour growth by altering the production of various interleukins or by activating protein kinases. They can also modify the cell cycle by inducing non apoptotic G1 phase mechanism, G2/M phase cell cycle arrest and apoptosis. It can downregulate telomerase activity and also inhibit angiogenesis. Thus lectin seems to have great potential as anticancer agent.

1.2. Description of jack-bean

Canavalia ensiformis is a legume crop, commonly known as jack bean which is used for animal food and human nutrition. It is a climbing perennial legume commonly cultivated as annual. The height of the plant is up to 2m with 8-20cm long trifoliate leaves. This plant has strong root system and the flowers are pink or white with a red base. Pods are up to 36 cm long and contain ellipsoid seeds which have 1-2cm long. Pods and seeds are edible and used for food. The whole plant is also used to feed animals.

1.3. Distribution

Jack bean was originated in Central America and west-indies. Now it is widely distributed in tropical and subtropical regions. This crop was cultivated in worldwide from united state, Brazil, Argentina, China and India. In Brazil it is called as “pig bean”. It was used for tropical soil reclamation efforts and avoiding the wide range of soil acidity, salinity condition. It has also capable of nitrogen fixation. Mainly jack beans were free from pets and disease. Jack bean has also reported as antagnostic or suppressive of nematodes. These legume plants are not cultivated commercially because the beans are mildy toxic in nature and copious

consumption must be avoided. Jack bean is the main source of concavalin A, a lectin used for several applications.

Classification of *Canavalia ensiformis*:

Kingdom	*Plantae*
Sub-Kingdom	Tracheobionta
Super-Division	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Sub-Class	Rosidae
Order	Fabales
Family	Fabaceae
Genus	Canavalia
Species	ensiformis

Table-1: Sytemetatic classification of Jackbean (*Canavalia ensiformis)*

Figure 1: Jackbean tree with pod

Figure 2: Jackbean seed

1.4. Concanavalin A

Concanavalin A (Con A) is a lectin or carbohydrate binding protein. It is a member of legume lectin family, originally extracted from the seeds of Jackbean (*Canavalia ensiformis*). It was a single protein having three dimensional structures. It binds to certain structures specifically found in various sugars, glycoproteins, and glycolipids, basically internal and terminal non reducing α-D-mannosyl and α-D-glucosyl groups of sugars ([4]*Goldstein and Poretz, 1986*). It was the first lectin to be available on a commercial basis. It is widely used in biology to characterize glycoproteins and other sugar binding proteins. It is also used to purify glycosylated macromolecules in lectin affinity chromatography in addition to study immune regulation by various immune cells.

1.5. Characteristics of Con A

Con A is a representative member of legume lectin class of plant protein. It is a homotetramer having subunit 26.5KDa and consists of 237 amino-acids residues, but it does not contain any cysteine residues ([5]*Olson and Liener 1967*). It binds with metallic atom usually Mn^{2+} and a Ca^{2+}. Con A interacts with metals on the molecular basis as well as its affinity for sugar molecules. Con A exists as a dimer or tetramer (*Loris et al, 1998*) and also exists as a dimetallised dimer. The molecular weight is about 104-112KDa. It is a plant mitogen and has the ability to activate mouse T-cell subsets which gives rise to four T-cell populations like precursor and suppressor T-cell ([6]*Powell and Leon 1970*). One subset of human suppressor T-cell is most sensitive to Con A. Con A can initiate cell division by stimulating the energy metabolism of thymocytes, principally acting on T- lymphocytes. It can also use in specific binding action with carbohydrate containing receptors.

1.6. Structure of Con A

Con A was the first lectin whose structure was known. It has a three-dimensional structure with tentative amino-acid sequence ([7]*Bruce A et al, 1972*). It is a homotetrameric subunit (26.5kDa). The protein consists of 237 glycated amino acids and has two metal binding sites

i.e. Ca^{2+} (an ion) or Mn^{2+} (a transition metal) (*Min W et al, 1972*). According to structural feature the amino acid chain contains two anti-

parallel beta sheets. Out of the two beta sheets one is built from seven bands and other one contains six strands. In its active state ConA forms aggregates. After joining two six stranded sheets resulting as a dimeric molecule (twelve stranded sheets). Two dimers form a functional complex by layering side to side. The final complex contains four spatially well separated binding sites for oligosaccharides.

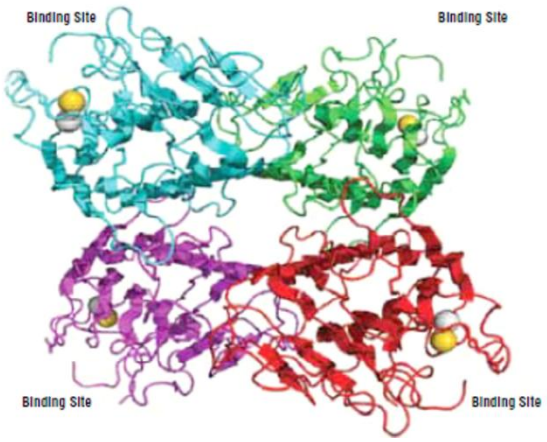

Figure 3: Homotetrameric structure of concanavalin A

1.7. Biological activities of Con A

The monomer of Con A is called as 'jelly roll' motif composed of two anti-parallel sheets. Con A was interacting with both carbohydrate containing mannose and also with rhodopsin, immunoglobulins, lipoproteins and carcino-embryonary antigen. It has been observed that tryptophan and tyrosine residues are involved in binding of carbohydrates. Preferably, it can agglutinate red blood cells and cancer cells. When transformed T cells and normal cells were treated with trypsin, they do not agglutinate at 4°C rather they agglutinate in the presence of con A. Joining of six strands form a diamer. Two diamers then form a complex by layering twelve stranded sheets. Thus four separate binding sites for oligosaccharides are formed and they are able to agglutinate cells like erythrocytes, myocytes, B-lymphocytes, fibroblasts,

human fetal, ratthymocytes, intestinal epithelial cells, adipocytes etc. Con A can stimulate several matrix metalloproteins (MMP) ([8]*Wecksler, M., et al., 1968*). It has a high agglutination activity towards cancerous cells or transformed cells but no agglutination effect in normal cells ([9]*Inbar and Sachs, 1969*). Excess amount of Con A can be harmful for the organisms because it is toxic in nature and toxicity of Con A can triggers apoptosis and autophagy in cell. By this mechanism, Con A is responsible for killing the cancer cells by inhibiting the activity of DNA polymerase alpha, by which the further replication process was blocked and then stopped cell division.

1.8. Application of Concanavalin A

Con A, the novel lectins have several applications in many aspects. The identification of first lectin is Con A, which is a macromolecular mitogen. Con A binds with saccharides by forming hydrogen bonds like other saccharide binding proteins (*Quiocho 1986*). Concanavalin A is also used to study for the relationship between cell toxicity and aggregation process. Con A has anti-proliferative activity on human cancer cells. It can also induce cancer cell death through apoptotic and autophagic pathway. In apoptosis process it lacks function of p53 and in autophagic pathway through mitochondrial process. It can induce human melanoma A375 cell in caspase-dependent manner that means in autophagic pathway (*Liu B et al, 2009*). It was recently reported that Con A has anti-hepatoma (liver cancer) effect ([10]*Lei HY, and Chang CP 2009*). Con A can use to immobilize the glycoenzymes, because in traditional covalent coupling they face several difficulties to immobilize them. It was used in immuno-electrophoresis for detecting the hormone associated variation in α1 acid glycoprotein (*Christine wells et al, 1980*).

2. REVIEW OF LITERATURE

Concanavalin A (ConA), the first well-known representative of legume lectin family, has increases the attention because of the interesting biology effects. Because of specific binding properties of carbohydrate, Con A has several aspects in several fields. Starting from 1916 to Now a Days there are several researches on Con A. Con A history was started from 1916 (*Jones, D.B.; Johns, C.O. ; 1916*). In this year isolation of two globulins from jack bean were studied. Then in 1936, by crystallization and purification it was identified that Con A was the first legume lectin. Further research on Con A in 1936 confirmed that lectins were carbohydrate-binding proteins and Con A was very specific for glucose and mannose ([12]*Sumner, J.B.; Howell, S.F.; 1936*). The primary and three-dimensional structures of Con A were resolved in 1972 (*Hardman, K.D.; Ainsworth, C.F.; 1972*). Then in 1982 it was demonstrated that Con A depend upon metal ions (*Hardman, K.D. et al., 1982*). Later in 1985, biosynthesis and molecular cloning of Con A was performed (*Carrington et al., 1985*). Con A induced apoptosis in fibroblasts was reported in 1995 and carbohydrate-binding property of Con A was intimately associated with apoptosis-inducing activities ([13]*Kulkarni et al., 1995*). Until 2007, it was demonstrated that Con A-induced autophagy through mitochondrial pathway (Fig.4).

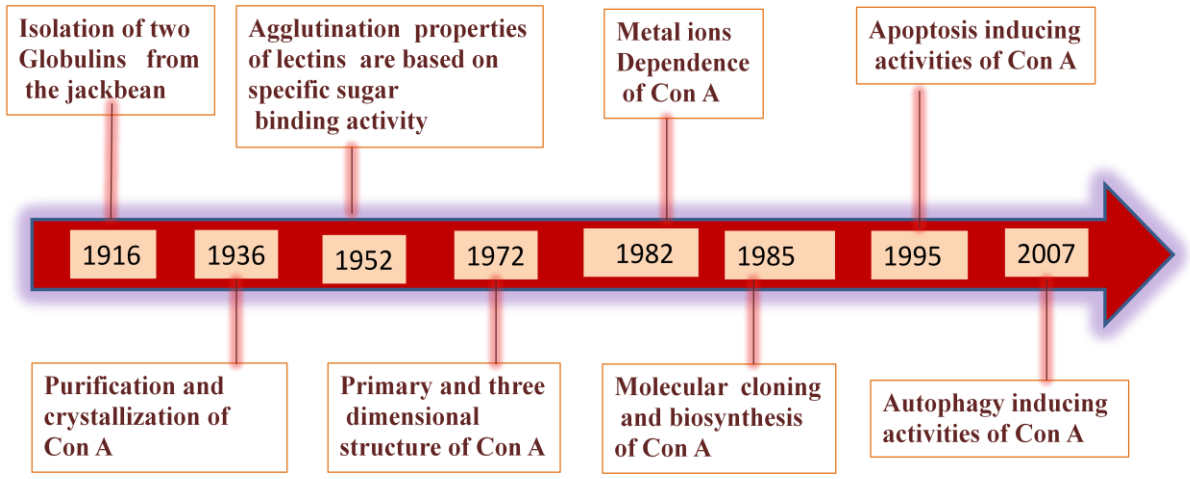

Figure-4: The timeline of Con A

Con A is a prospective anti-neoplastic agent for cancer therapeutics by targeting apoptosis and autophagy. Apoptosis, the self-killing process and autophagy, the self-eating process are two important cell death mechanisms.

2.1. Programmed Cell Death:

Programmed cell death (PCD) is a death of a cell by any form in a regulated process. PCD is an evolutionarily conserved process which helps to decide about cell fate. PCD is a intrinsic mechanism of cells which plays a vital role for eliminating harmful cells and maintaining homeostasis. Mainly there are two forms of PCD i.e. apoptosis and autophagy. There are two types of PCD, type I (apoptotic cell death) and type II (autophagic cell death). There are also several other mechanisms such as necrosis, msenescence, and mitotic catastrophe.

I. Type I Programmed Cell Death.

The type-I apoptotic cell death is a form of PCD characterised by several morphological features such as chromatin condensation, nuclear fragmentation, membrane blebbing, cell shrinkage and apoptotic body formation. Apoptosis is a caspase dependent process. There are two main pathways in apoptosis, extrinsic and intrinsic. Extrinsic pathway is a receptor mediated pathway where as intrinsic pathway is a mitochondrial mediated pathway.

II. Type II Programmed Cell Death.

Apoptosis, the type-II programmed cell death is a catabolic process which involves degradation of cells own component by lysosomal process. Mechanism of autophagy involves isolation of membrane formation then it identifies the cellular components for degradation. By this process autophagosome formation comletes and fuses with lysosome to form autophago-lysosome or autolysosome which degrades the cellular components.

The above three mechanisms apoptosis, autophagy, and necrosis are being interconnected with each other. ([14]*Vassiliki Nikoletopoulou et al., 2013*). Interaction between these three mechanism control whether a cell lives or dies (fig-3). Any de-regulation of this mechanism may lead to several diseases and it may also alter the characteristics of cell death.

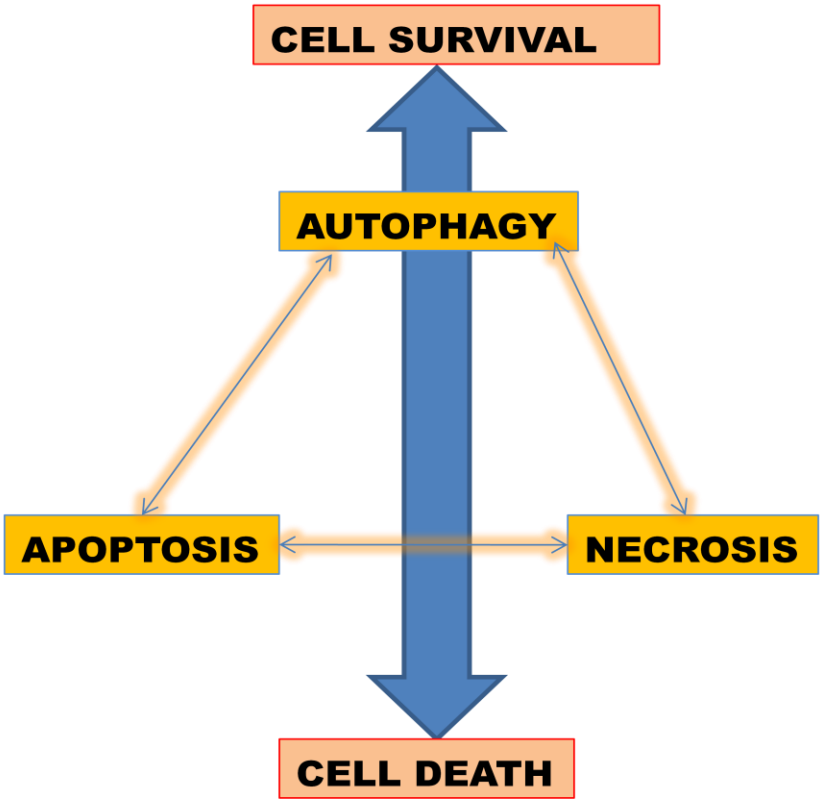

Figure-5: Cell death mechanism

2.2. Autophagy

Autophagy, the "self eating" process is a cell death mechanism, which degrades the unnecessary components of cells. Autophagy is a regulated process of degradation and recycling of cellular constituents and it can also participate in organelle turnover. It serves as

a protective role by allowing cells to survive during nutrient starvation. It is an important mechanism of cellular self-degradation. Autophagy plays an essential role in cellular and whole-animal homeostasis and differentiation. Autophagy, a dynamic pathway is controlled by several genes and the genes are connected each other by autophagosome formation. It is a nonselective degradation process which directly engulfs the cytoplasmic organelles ([15]*Johansen and Lamark 2011*). Deregulation of autophagy may contribute many diseases like ageing, neuro-degeneration, tumour, heart disease, lung and liver diseases and many other diseases ([16]*Levine and Kroemer 2008; Mizushima and Yoshimori 2007*). Therefore it is important to characterize this process at the molecular level.

2.3. Types of autophagy

There are mainly 3 types of autophagy such as microautophagy, macroautophagy and chaperone-mediated autophagy (CMA).

CMA is the mechanism which allows the degradation of cytosolic proteins and organelles.

In this process, first the soluble cytosolic proteins are targeted to lysosomes, then removes through lysosomal membrane by translocation. Mostly CMA is studied in the mammalian cells for concerning pathogenesis of different diseases. This is activated in response of stress like oxidative stress and exposure to toxic materials ([17]*Kaushik and Maria 2012*).

Macroautophagy is a vital process in the eukaryotic cells. This process controls the arrangement and sequestration of the cellular components which need to be recycled by lysosome (*Kanazawa et al. 2003; Wang and Klionsky 2003*). In mammalian cells, this process is activated by lower insulin level ([18]*Mortimore et al. 1989; Mortimore and Poso 1987; Seglen and Bohley. 1992*). Previously it is reported that the micro- or macro-autophagy can occur both in the same organism and also same time, such as the mitochondrial selective degradation (*Kissová et al. 2007*). Macroautophagy simply referred as autophagy which is a lysosomal degradation pathway.

Microautophagy is described as a nonselective lysosomal degradative process (*Dice, 2007*). It is a form of autophagy involving turnover of long-lived proteins, while substrate soluble microautophagy can be activated by rapamycin through regulatory signaling complex pathways (*Dalibor et al. 2011*).

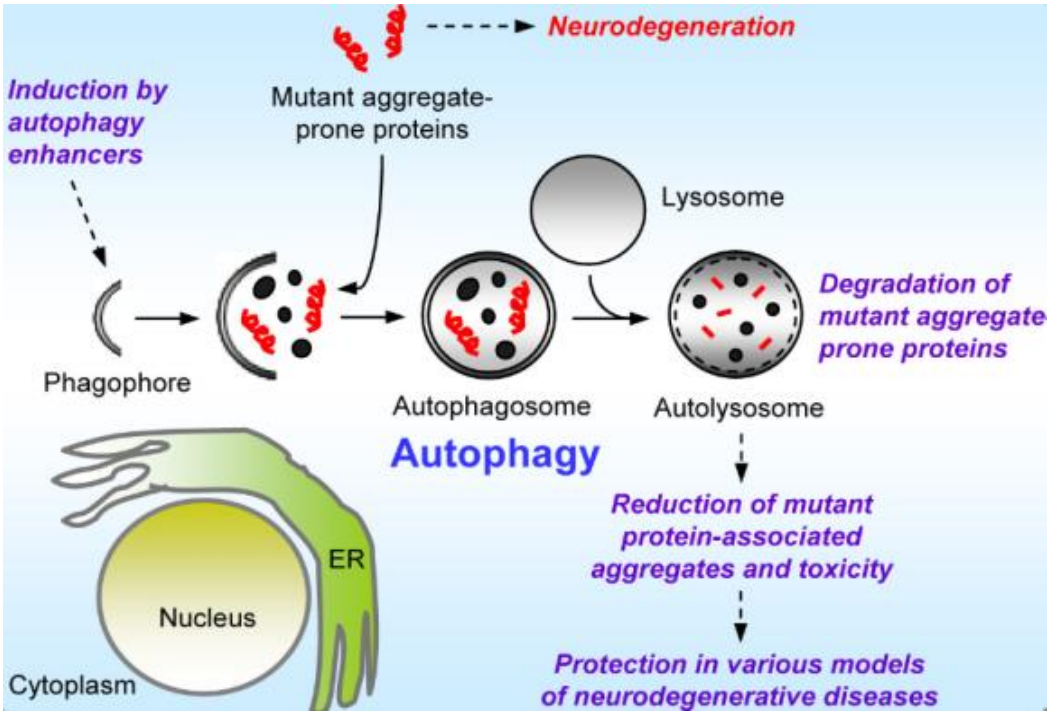

Figure-6: Autophagy mechanism

2.4. Autophagy and cancer

Autophagy plays an important role in cancer. Cancer is a generic disease having uncontrolled proliferation of cells. Autophagy is a critical mechanism for cancer cells because these cells required large amount of energy for cell growth ([19]*Levine, B., 2007*). On one hand, deregulation of autophagy may lead to genomic instability and this process promotes the tumour initiation and progression of autonomous tumour cells. In other case autophagy defects may lead to several alternations in cells like tumour cell survival in stress condition, necrotic cell death, inflammation etc. Cytokines are generated through inflammation which

helps in promoting tumor cell growth. Hence autophagy acting an important role both in case of cell survival and the death of the cells ([20]*Liu, B et al., 2009*).

2.5. Autophagic pathway for cancer therapy

There are several methods for prevention of cancer by targeting the autophagy pathway. If we can identify autophagic pathway which is required for the suppression of tumorigenesis, then for cancer therapy autophagy can act as a new target.

I. Inhibitor of autophagy blocks metabolic stress of cell survival

Autophagy inhibitors can target tumour cells in hypoxic tumour regions, so that these are particularly attractive for cancer therapy. Tumour cells may also particularly dependent on autophagy in the process of metastasizing.

II. Inhibition of apoptosis

An alternate approach for cancer therapy is the inhibition of apoptosis. There are various protein families which plays major role in inhibiting apoptosis. Apoptotic inhibitors are class of proteins which inhibits apoptosis. For example: Bcl-2 is a apoptotic inhibitor which inhibits apoptosis. For this mechanism Bcl-2 requires Bim, the pro-apoptotic protein. Additionally Bax and Bak are required for this process. So these apoptotic inhibitors plays a novel role in anti-cancer properties.

III. By developing autophagic inhibitors

Activation of autophagy and autophagosome formation is regulated by kinases (Atg1/Unc-51, vsp34), proteases (Atg4) and two ubiquitin like conjugation system. There are also signalling pathway that regulate activation of autophagy i.e. both mTOR-dependent and –independent (mammalian target of rapamycin). For autophagy inhibitor development, signalling pathway are good candidates, because they promote autophagy. For example, hydroxychloroquine

(HCQ) is an autophagy inhibitor, which blocks lysosome acidification and autophagosome degradation, which enable assessment.

2.6. CROSS TALK BETWEEN AUTOPHAGY AND APOPTOSIS:

Apoptosis, a programmed cell death is characterized by cell death through morphological changes, including shrinkage of nucleus, fragmentation, chromosomal DNA fragmentation, and chromatin condensation. Number of diseases results in the defective apoptosis like atrophy, which is the result of excessive apoptosis. But in cancer the insufficient amount results unrestrained cell proliferation. Apoptosis and autophagy are connected with each other by several processes. Autophagy can provide a foundation for apoptosis while the pathway of apoptosis can be delayed by the inhibition of autophagy ([21]*ShuYan et al. 2014*). Autophagy and apoptosis are two important cellular processes having a complex protein networks. For example, Bcl-2 is a protein which acts as a central regulator for both apoptosis and autophagy mechanism (Table -2).

Bcl-2 inhibits autophagy when it is localized to the endoplasmic reticulum and mitochondria. Autophagy and apoptosis are triggered by the displacement of Bcl-2 from Beclin-1during Cellular stress. BH3-only proteins and post-translational modification can disrupt the complex through autophagy and apoptosis mechanism ([22]*Chun-Yang Li et al., 2010*). There are several autophagic proteins which are which regulate both extrinsic as well as intrinsic apoptosis. For instance, calpain- and caspase-mediated cleavage of autophagy-related proteins can switches the cellular program from autophagy to apoptosis. This highlights a dual cellular role for autophagy. Autophagy degrades caspases and the damaged mitochondria. It also provides a membrane-based platform for caspase processing for apoptosis regulation. The crosstalk between autophagy and apoptosis is complex, and this crosstalk is critical to cellular fate. In certain cellular conditions, autophagy can promote cell

survival and avert apoptosis, and in some other cases autophagy may lead to cell death ([11]*Huan-Yao Lei* and Chih-Peng Chang ., 2009*).

Table-2: Role of proteins in both autophagy and apoptosis (*Mukhopadhyay, S., et al., 2014*).

PROTEIN	ROLE IN AUTOPHAGY	ROLE IN APOPTOSIS
AUTOPHAGIC PROTEINS		
mTOR	Inactive form involves in initiation	mTOR regulates apoptosis
Beclin-1	Autophagosome nucleation	Cleaved C-fragment induces mitochondrial apoptosis
UVRAG	Upregulates Vps34–Beclin1 interaction	Antiapoptotic, inhibits Bax translocation from cytosol to mitochondria
AMBRA	Upregulates Vps34–Beclin1 interaction	Regulate mitochondrial apoptosis; cleaved by caspases and calpains
Atg3	Conjugates with Atg12	Regulates mitochondrial cell death
Atg5	Conjugates with Atg12, autophagosome elongation	Interacts with FADD to inhibit apoptosis, cleaved N-fragment induces mitochondrial apoptosis
Atg12	Autophagosome elongation	Stimulates mitochondrial apoptosis by inactivating Bcl-2 and Mcl-1
Atg4D	LC3 processing	Cleaved Atg4D localize to mitochondria and induces apoptosis
APOPTOTIC PROTEINS		
Bcl-2	Inhibitor of autophagy	Anti-apoptotic
Bad, Bak,	Pro-autophagic, disrupting Beclin-1/Bcl-2 interaction	Pro-apoptotic

BNIP3, Nix		
Bax, PUMA	Proautphagic, noncanonical type	Pro-apoptotic
p53	Inhibits by cytoplasmic p53	Pro-apoptotic
Noxa	Induces autophagy by disrupting Mcl-1/Beclin-1 interaction	Pro-apoptotic
Bim	Sequesters Beclin-1, inhibits autophagy	Pro-apoptotic
XIAP	Inhibits by Mdm2-p53 signalling	Inhibits caspase 3,7
cFLIP	Prevent interaction between Atg3 and LC3	Inhibits casepase 8

2.7. Con A induced Autophagy and related molecular mechanisms

Previously it was reported that Con A has specific sugar binding residues, this property helps to induce cell death. In case of tumour cell lines, cytotoxic casues at high concentration where as at low concentration it is cytoatatic ([23]*Rubinsztein et al., 2007*). After binding with mannose moiety, Con A was internalized to the mitochondria, and then the autophagic cell death was initiated through clathrin-mediated endocytosis ([24]*Lei, H.Y. et al., 2009*).

It was shown that Con A treatment with CT-26, ML-1 cell line there were many autophagic pathway observed, these are, formation of LC3-II, vesicles with double-layer and induction of BNIP3. In case of ML-1 cells it noticed that autolysosome formation was takes place which indicates that Con A induced autophagy ([25]*Lei, H.Y. et al., 2007*). Autophagy induced by Con A is an autophagic flux. It was observed that in hepatocellular cells Con A induced autophagy. But these were not observed in oral cancer cell lines (*Chang, C.P. et al., 2007*). Apart from this, Con A also serves as T cell mitogen modulating immune system. Production of cytokine inside the tumor was activated when Con A induced immune system in hepatoma cell. Then lymphocytes were needed, which further gave rise to the elimination of the tumor.

2.8. Con A induced apoptosis and related molecular mechanisms

From previous report, it was found that Con A induce apoptotic cell death ([26]*Chen, M et al., 2002*). It was indicated that Con A at this concentration acted as T-lymphocytes and thymocytes mitogen ([27]*Cribbs, D.H. et al., 1996*). Con A was specifically binds with lectin and receptor glycoprotein. When neurons treated with Con A there was seen many morphological changes in the cell, including membrane blebbing, chromatin condensation and many others. These changes were later proved as typical features of apoptosis (*Cribbs, D.H. et al., 1996).* After ConA treatment this experiment also observed increase in immunoreactivity for c-Jun [36]. Now c-Jun involved in the process of apoptosis ([28]*Krens, S.F. et al., 2006*). When central nervous system is injured, the astrocytes in human brain can form glial scar to prevent neuronal regeneration (*Gabryel, B. et al., 2001*). Con A was exposed to bind with astrocytes and triggering apoptotic cell death. This binding occurs due to the carbohydrate binding activity of Con A ([29]*Yoshida, H. Et al., 2009*). These findings provide possible mechanism for treating injured nervous system through mediation of apoptosis.

Another research reported that Con A has anti-proliferative activity. Con A was positively correlates with its sugar-binding and hemagglutination activitie (*Liu, Z.Y. et al., 2010*). A tumour suppressor gene, p53 regulates cell cycle and apoptosis. But it is often mutant in cancer cells ([30]*Harris, S.L. et al., 2005*). Further it was indicated that Con A treatment caused an initial G2/M delay followed by G1 arrest (*Amin, A.R. et al., 2007*). Lacking p53 cells undergo apoptosis, and the cells with p53 were protected by arresting in G1 phase. It was also investigated that Con A-induced expression of p73 was independent on E2F1. But it was regulated by reactive oxygen species (ROS) and for the first time confirmed that Con A could

activate p21, cdk inhibitor through p73-dependent manner (*Amin, A.R. et al., 2010*). In tumor cells, the induction of apoptotic cell death would serve as an effective cancer therapy agent. Because of this remarkable apoptosis-inducing activities Con A has been drawing rising attention towards the autophagy and apoptosis.

2.9. Con A has anti-proliferative activity

Con A has been drawing rising attention for its potential applications in cancer therapeutics. Recent studies shows that Con A with mannose/glucose specificity has anti-tumour activity. Further experiment on Con A proved that the antiproliferative activity was influenced by hemagglutination ([31]*Bo Liu et al., 2009*). Con A has specific sugar binding activity which overlaps the hemagglutinating active centre and it was indicated by the carbohydrate-binding activity assay. Hence, Con A shows a major effect in anti-proliferative activity due to its carbohydrate binding nature.

2.10. Chemoprevention nature of Con A

Con A is toxic in nature and its toxicity property can triggers both autophagy and apoptosis in cancer cells. In case of animals when lectins are orally injected, it causes intoxification ([32]*Liener IE., 1995*). There are several plant extracts which are used in adjuvant cancer therapy by injected it. For instance, in Europe Mistletoe, the galactoside-specific lectin is used for the alternative treatment for cancer. In case of soybeans, there are many anti-carcinogenic phytochemicals are present. ([33]*Schumacher K et al., 2003; de Mejia EG et al., 2003*). Previously it was also reported that in boby system Con A can inhibits liver nodule formation by oral injection. This indicates the chemopreventive nature of Con A for cancer treatment ([34]*Rosenberg SA et al., 2004*). Con A-like substances, particularly natural substances like edible vegetable plants, seeds and health foods have both immunomodulating and autophagy-inducing activities.

OBJECTIVES

1. To determine the morphological changes of Hep-2 cells by

   Bright field microscope

   Tryphan blue Exclusion Assay

2. To study con A induce autophagy in Hep-2 cells by

   Acridine orange staining

3. To determine apoptotic cell death by

   DAPI staining

   Annexin V FITC staining

   Caspase-3/7 Glo Assay

4. To study the relationship between autophagy and apoptosis

   by Chloroquine treatment

3. MATERIALS AND METHODS

3.1. SAMPLE COLLECTION:

The *Canavalia ensiformis* (jack-bean) seeds were collected from Angul, Odisha, India. Con A, a lectin from jack bean seeds were previously extracted in the laboratory of Cell and Cancer Developmental Biology (CCD-lab), NIT Rourkla, Odisha, India.

3.2. **CHEMICALS**:

Sodium carbonate (Na_2CO_3), Sodium hydroxide (NaOH), Cupper sulphate ($CuSO_4$), Glycine, Potassium sodium tartarate ($KNaC_4H_4O_6$) were purchased from SRL, Sisco research laboratories Pvt. Ltd., Mumbai. Ammonium per sulphate (APS), Acrylamide, bisacrylamide, Sodium dodecyl sulphate (SDS), ethanol, Bovine serum albumin (BSA), N, N, N', N'-tetramethylenediamine (TEMED), and Tris were purchased from Sigma Aldrich, USA. Potassium hydrogen phosphates (K_2HPO_4), Potassium Dihydrogen Phosphate (KH_2PO_4), were purchased from S.D. fine chem. Ltd., Mumbai. Glycerol was purchased from RANKEM Pvt Ltd. Ethanol was from Trimurty Chemicals, India. Acetic acid, Bromophenol blue, Typhan blue and agarose were purchased from Himedia, Mumbai. Cell culture media viz. MEM, DMEM were purched from the Gibco. Silver nitrates, Sodium thiosulphate, Methanol, were purchased from nice chemicals Pvt .Ltd. India. Pre stained molecular weight marker were purchased from Bio-Rad, India. Trypsin & antibiotics were purchased from the Hi-media. Acridine Orange, DAPI and Annexin V-FITC were purchased from the Sigma. T25 and T75 cell culture plates were purchased from tarson, India.

3.3. Cell line:

The whole experiment was carried out by using the oral cancer cell line HEp-2, which was collected from National Center for Cell Science (NCCS), Pune.

3.4. Cell viability assay:

Principle:

Cell Viability is the test to determine the ability of cells to maintain its viability. There are several assays or test used to determine the cell viability. For example: by using propidium iodide (PI), typan blue etc. Here, simply using bright field microscopice, the number of viable cells was determined.

Methods:

Preseeded HEp-2 cells were treated with Con A and kept incubation for 24 hrs. After that visualisation study was done under microscope.

3.5. Trypan blue exclusion assay:

Principle:

The trypan blue exclusion test is used to determine the number of viable cells present in a cell suspension. The principle behind this test is that live cells exclude certain dyes like trypan blue because of intact cell membrane. But the dead cells take up the dye because of inability to exclude the dye. This test is used to determine whether the cells either take up the dye or not.

Methods:

Cell suspension aliquot was tested for viability for 5 min at 100×g. Then it was centrifuged and supernatant was discarded. Aliquot size depends upon the number of cells present in that cell suspension. 0.4% trypan blue was mixed with 1 part of the cell suspension. Then observe under microscope. The viable cells have a clear cytoplasm where as the non-viable cells have a blue cytoplasm. The percentage of cell viability is determined by counting both the viable cells and non-viable cells.

3.6. Acridine orange staining

Principle:

Acridine orange (AO) is a nucleic acid selective fluorescent cationic dye. AO is useful for cell-cycle determination and it is cell permeable. It interacts with both DNA and RNA. It crosses into lysosomes and other acidic compartments and becomes protonated. AO is used in autophagic assays and is characterized by rising formation of AVOs (acidic vesicular organells) viz. lysosomes and autophagolysosomes. After staining the cells with AO, AVOs can be quantified by flow cytometry. AO is a weak base that accumulates in acidic spaces and produce red fluorescence. AVOs can be quantified with increasing the intensity of red fluoresce which is proportional to the degree of acidity.

Methods:

Cells were seeded in a 12-well plate. Then cells were treated with different concentrations of Con A for 24 hours. After that, acridine orange (AO) was added at a final concentration of 0.5µg/ml for 15 min. Then observation was carried out under the fluorescence microscope (Olympus IX_{71}).

3.7. DAPI staining:

Principle:

4', 6-diamidino-2-phenylindole, DAPI is a fluorescent stain. It binds strongly to A-T rich regions in DNA. DAPI, the nucleic acid stain preferably stains dsDNA. DAPI can pass through an intact cell membrane, so that, it can be used to stain both fixed and live cells. DAPI, the blue fluroscent probe, fluoresces brightly upon the minor groove of dsDNA and its fluroscence is approximately 20-fold greater than the non bound state. At 460 nm, the DAPI shows fluorescence in dsDNA. High cell permeability allows efficient DAPI staining of nuclei with the little background of cytoplasm. For immunofluroscence microscope it is a

classic nuclear counter stain. Cells undergoing apoptosis shows several features such as reduction in cell-size, chromatin condensation, membrane blebbing, and nuclear shrinkage. Formation of apoptotic body by this process can be detected under the fluorescence microscope using appropriate staining of nuclei with DAPI. The effectiveness of this stain is less because in live cells the efficiency of passing through the membrane is less.

Methods:

Preseeded cells were treated with con A at various concentrations followed by incubation for 24 hrs. Then the old media was discarded and cells were treated with paraformaldehyde for 30 minutes at 37^0C. Then washing with PBST and PBS was done for twice. In next step DAPI was added to the cells in dark room and incubated for 5 minutes. Again the cells were washed with PBS for three times. Then samples were studied under Olympus $1X_{71}$ microscope.

3.8. Annexin V-FITC staining

Principle:

FITC Annexin V staining can identify apoptosis at an earlier stage. Apoptosis is a normal physiologic process for removal of unwanted cells and it maintains tissue homeostasis. There are certain morphological features which are seen in case of apoptotic programme such as loss of plasma membrane asymmetry, chromatin condensation and nuclear shrinkage. From the above features, in apoptotic cells loss of plasma membrane was seen earliest. The membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane of apoptotic cells. Thus, it exposes PS to the external cellular environment. Annexin V is a Ca^{2+} dependent phospholipid-binding protein having molecular weight 35-36 kDa. It has a high affinity for PS, and binds the cells with exposing PS. Annexin V may be conjugated with Fluroescien Isothiocyanate (FITC) to level PS on membrane surface. FITC Annexin V causes the loss of membrane integrity and by which cell

death occures by two processes apoptosis and necrosis. Hence, FITC Annexin V staining is usually used in combination with a vital dye like propidium iodide (PI). This process allows the researchers to identify easily the apoptotic cells and the results will be PI negative, FITC Annexin V positive.

Methods:

Cells were seeded in 12-well plates and treated with different concentrations Con A and kept for 24 hrs. Then the cells were washed with PBS and 1X Annexin V binding buffer was added followed by addition of Annexin V-FITC stain and incubated for 15 mins. Then observed in fluorescence microscope.

3.9. Caspase-3/7 Glo Assay:

Principle:

The Caspase- 3/7 Glo Assay is a homogeneous, luminescent assay which measures caspase-3 and -7 activities. Caspase undergoes the member of the cysteine aspartic acid-specific protease family, which plays a vital role in apoptosis in case of mammalian cells. Caspase-3/7 substrate contains the tetrapeptide sequence DEVD. Amount of caspase activity present is proportional to the Luminescence. The Caspase- 3/7 Glo Reagent relies on the properties of a thermo-stable luciferase enzyme, which is formulated to generate a stable "glow-type" luminescent signal and improves performance across a wide range of assay conditions. The Caspase Glo-3/7 Assay is showing caspase activity or apoptosis.

METHODS

In this assay total volume of 100µl used in 1:1 ratio of caspase-3/7 Glo reagent volume to sample volume. The substrates required for this assay are proteins, lysis buffer and caspase-3/7 reagent. Protein was estimated by barford's method. After estimated 25µg protein was mixed with cell lysis buffer. Then caspase-3/7 Glo reagent was mixed with the solution and

makes the volume 100µl. After 6-8 hrs incubation in dark reading was taken in promega 20/20 luminometer.

4. RESULTS AND DISCUSSIONS

4.1. Cell viability assay

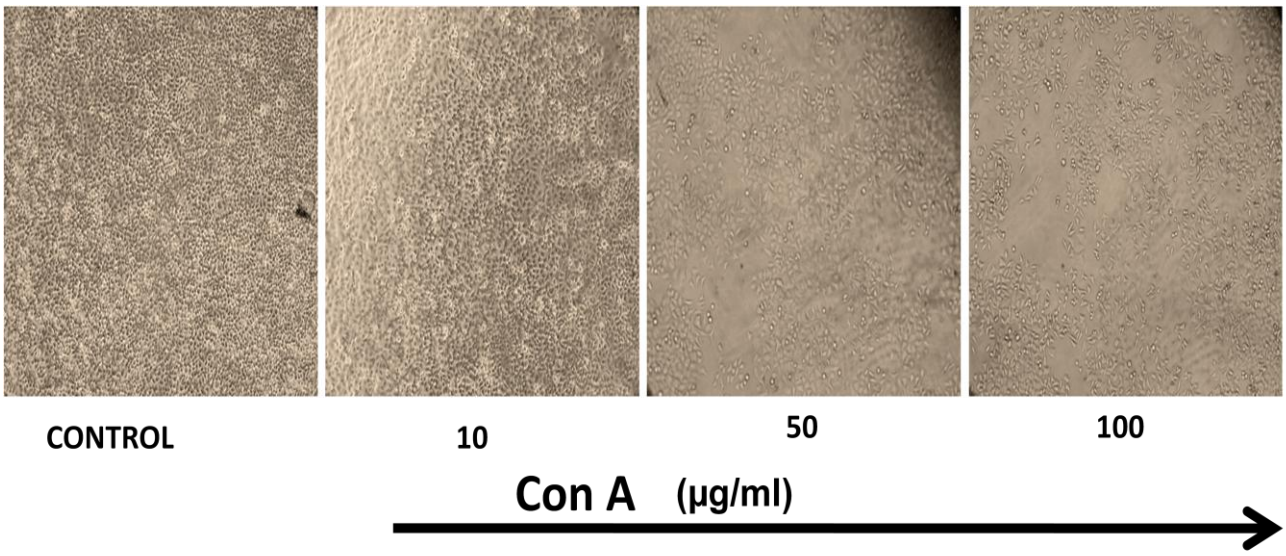

Fig-7: Showing cell viability test

INFERENCE

From the above figure it was inferred that as the concentration of Con A increases in a dose dependent manner in Hep-2 cells, the number of cell death increases accordingly, So that the number of viable cells decreases in a dose dependent manner.

4.2. Trypan blue exclusion assay

INFERENCE:

In this trypan blue exclusion essay (fig. 8), the cytoplasms of non-viable cells are in blue colour because trypan blue is being taken by these cells. But the viable cells have clear cytoplasm because the cells don't take up the dye due to cytotoxicity nature of Con A. Thus it was shown that the % of viability of Hep-2 cells decreases in a dose dependent manner with

Con A treatment (Table-2). The % of cell viability was calculated by the no. Of viable cells divided with total no. Of cells then multiply with 100. (Fig-8)

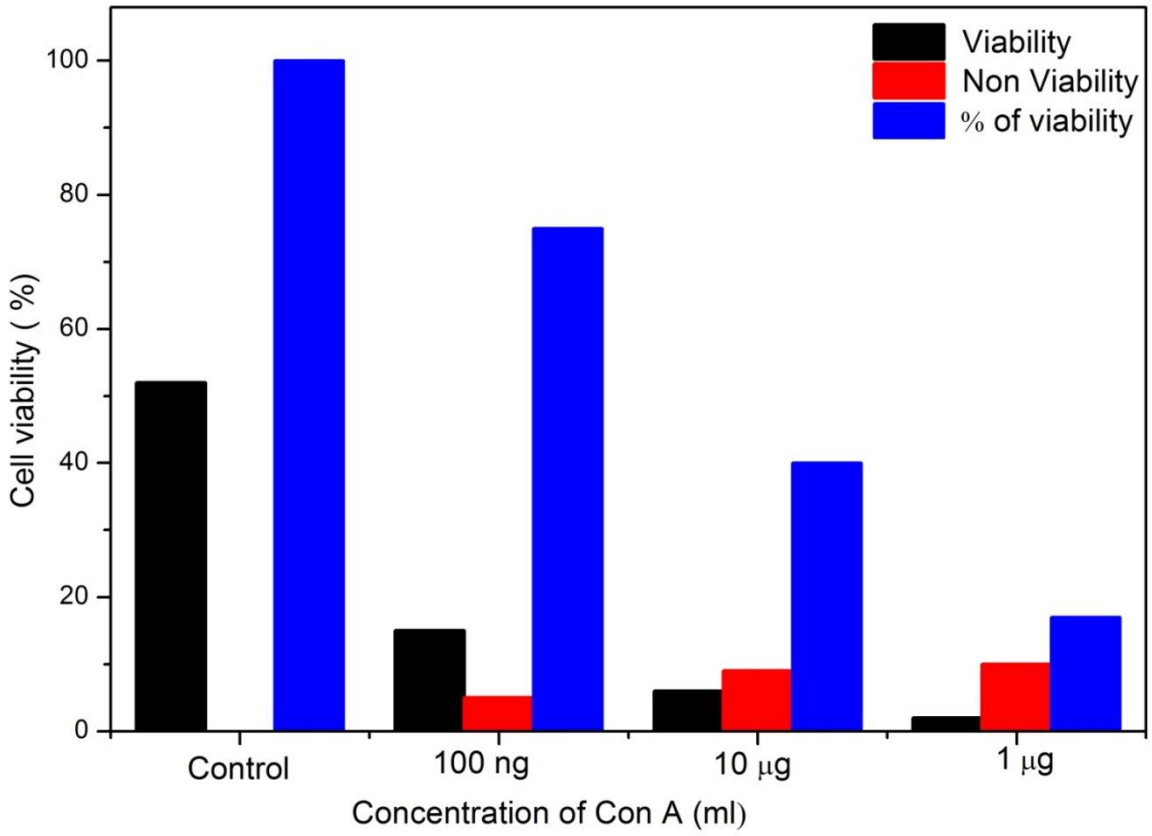

Fig-8: Graph showing % of viable Hep-2 cells after Con A treatment

4.3. Acridine orange staining

INFERENCE

It was inferred that when Hep-2 cells are treated with different concentrations of con A in a dose dependent manner, the redness level increases accordingly. The shift of redness increases with increase the dose of Con A which indicates the formation of autolysosome. So in higher dose i.e. in 100µg/ml there is more redness level which shows more autophagy in Hep-2 cells (Fig-9).

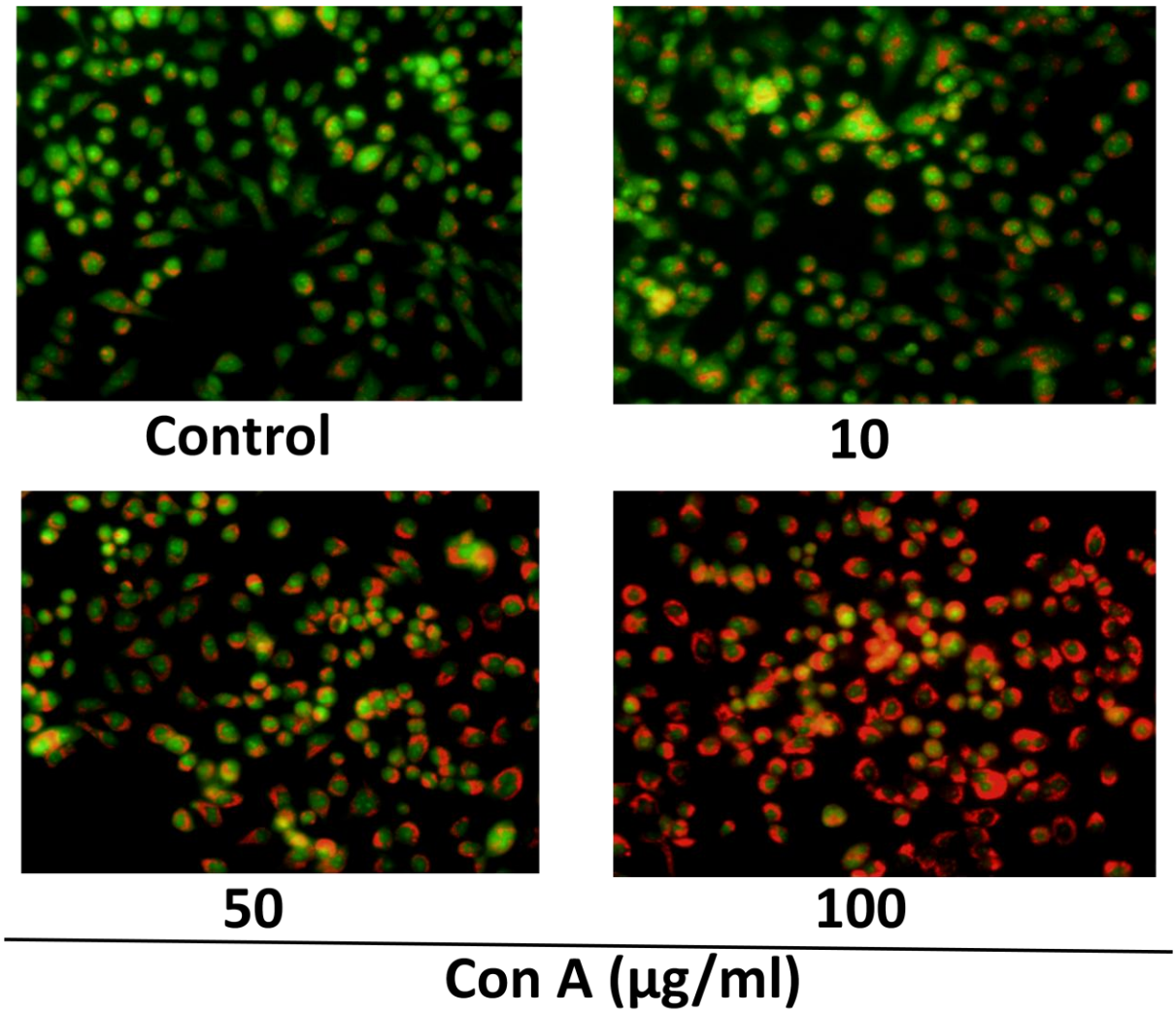

Fig-9: Showing acridine orange staining in Hep-2 cells after Con A treatment

4.4. DAPI Staining

INFERENCE

As the dose of the Con A increases in Hep-2 cells it was seen that the chromatin condensation and nuclear fragmentation also increases. At higher dose (100µg/ml) con A, more nuclear changes occur which depicting the apoptosis. (Fig-10).

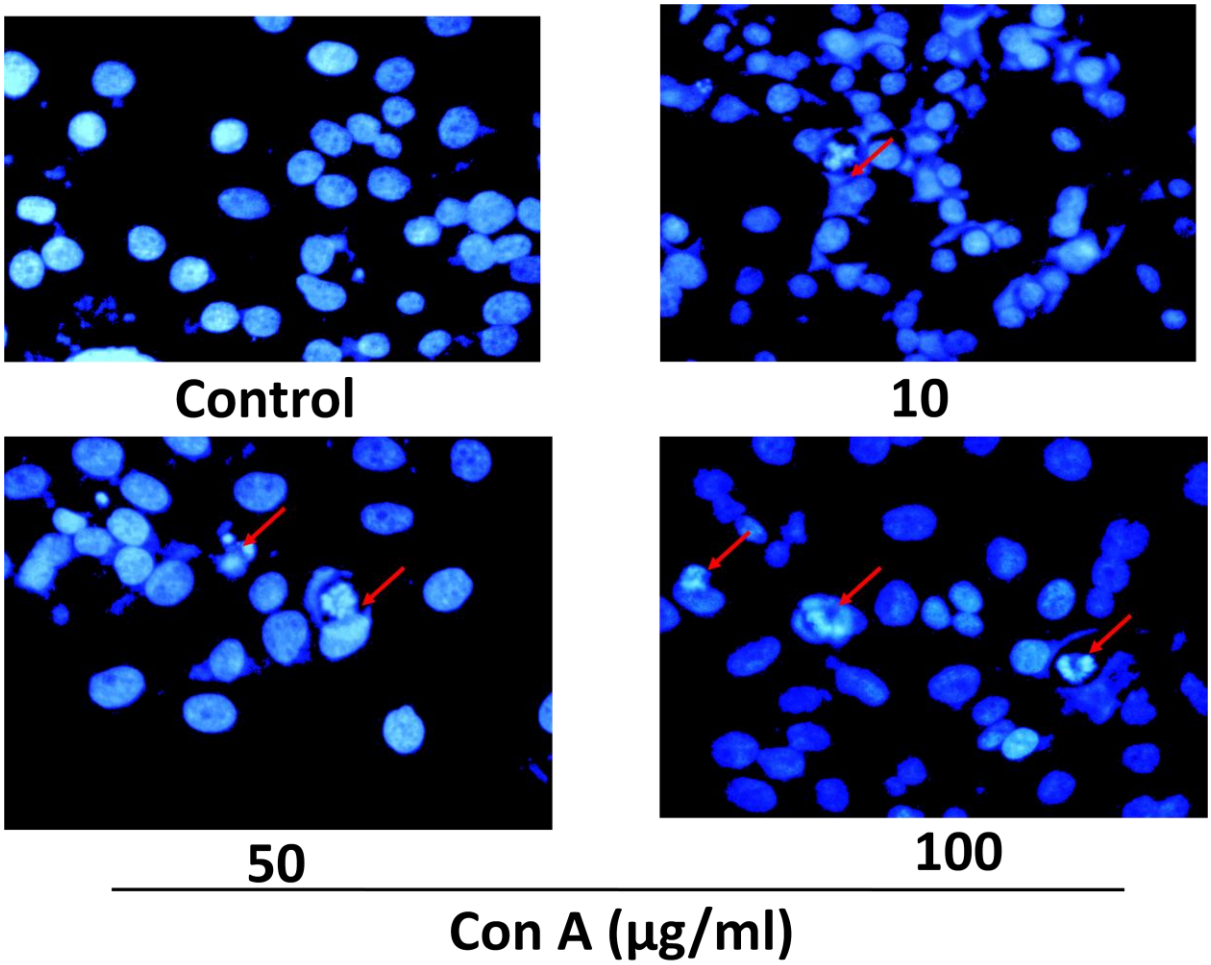

Fig-10: DAPI staining showing nuclear changes

4.5.Annexin V-FITC Staining

INFERENCE

Annexin V conjugates with FITC (flurophore molecule) binds with phosphotidyl serine (PS) and causes loss of membrane integrity which leads to apoptosis. From this figure it is observed that at higher concentration there is more membrane blebbing which shows more apoptosis. (Fig-11)

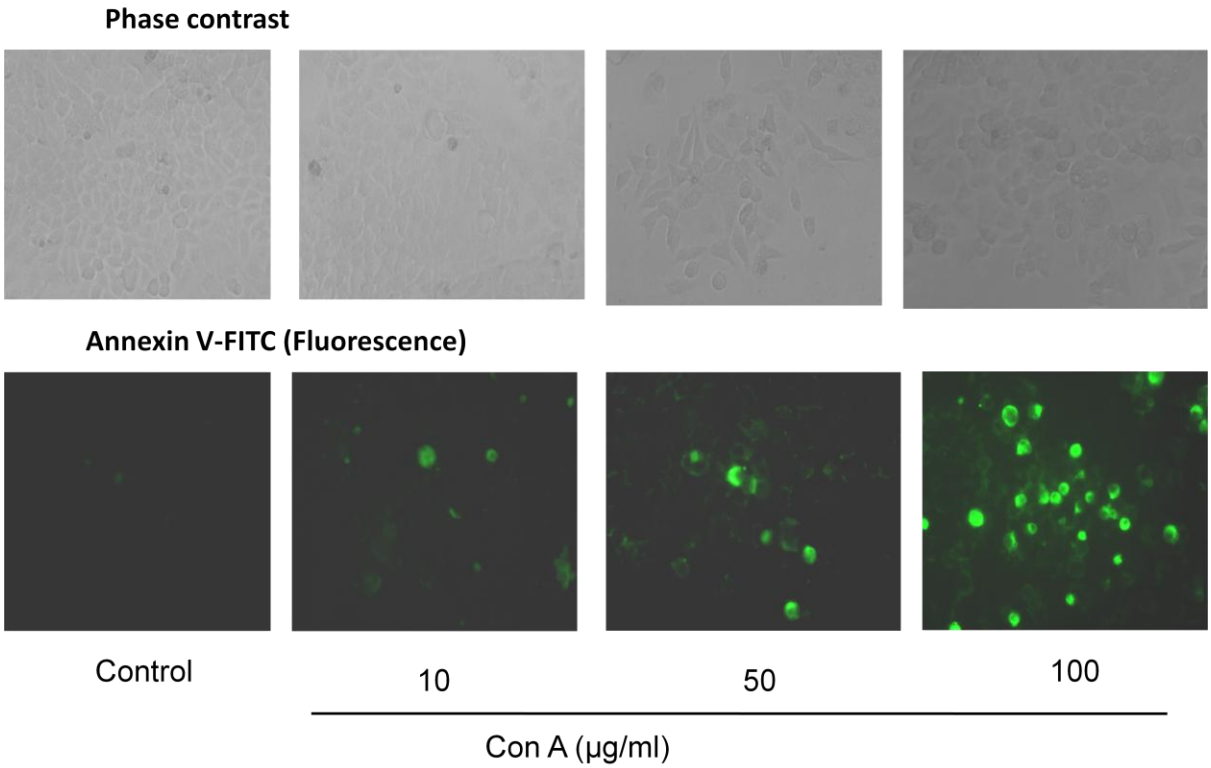

Fig-11: Showing Annexin V-FITC staining in Hep-2 cell

4.6. Caspase-3/7 Glo assay

INFERENCE

Apoptosis is a caspase dependent process, so that caspase-3/7 glo assay plays a major role in showing apoptosis. It was observed that the caspase activity increases accordingly with the increase of concentration of Con A. At higher concentration of con A i.e. 100µg/ml, more caspase activity is observed, which indicates more apoptosis. (Fig-12)

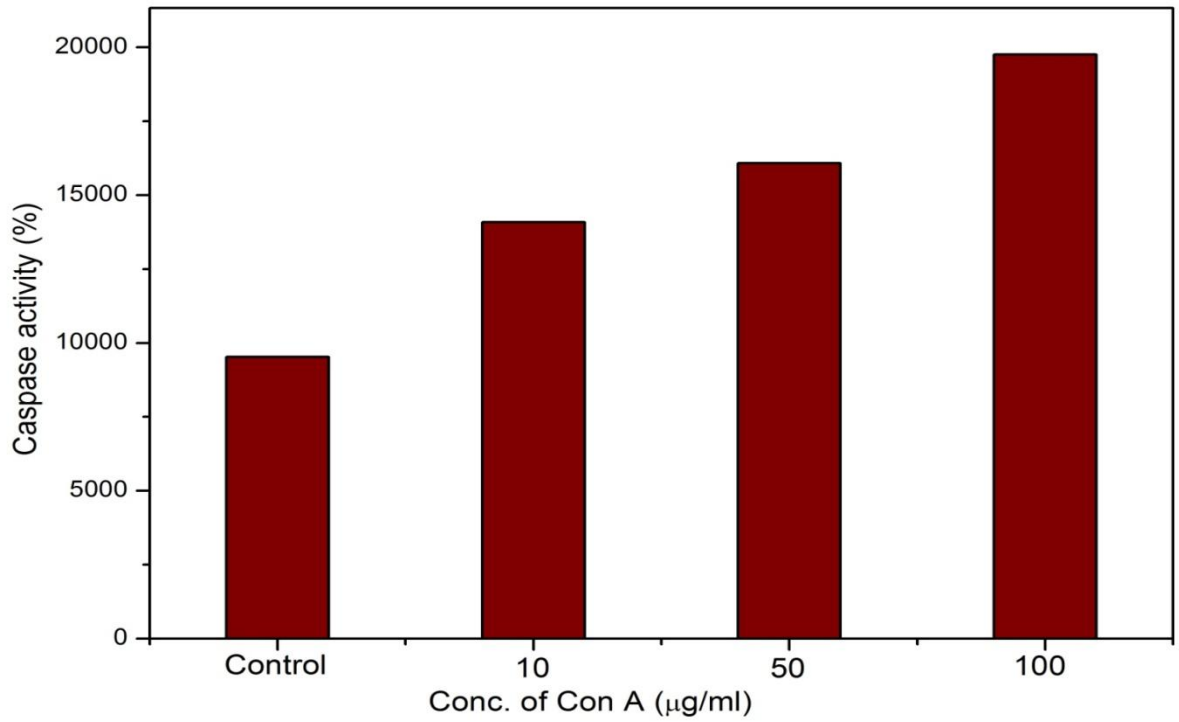

Fig-12: Graph showing % of caspase activity in Hep-2 cell post Con A treatment

4.7. Inhibition of autophagy by chloroquine inhibits Con A induced cell death

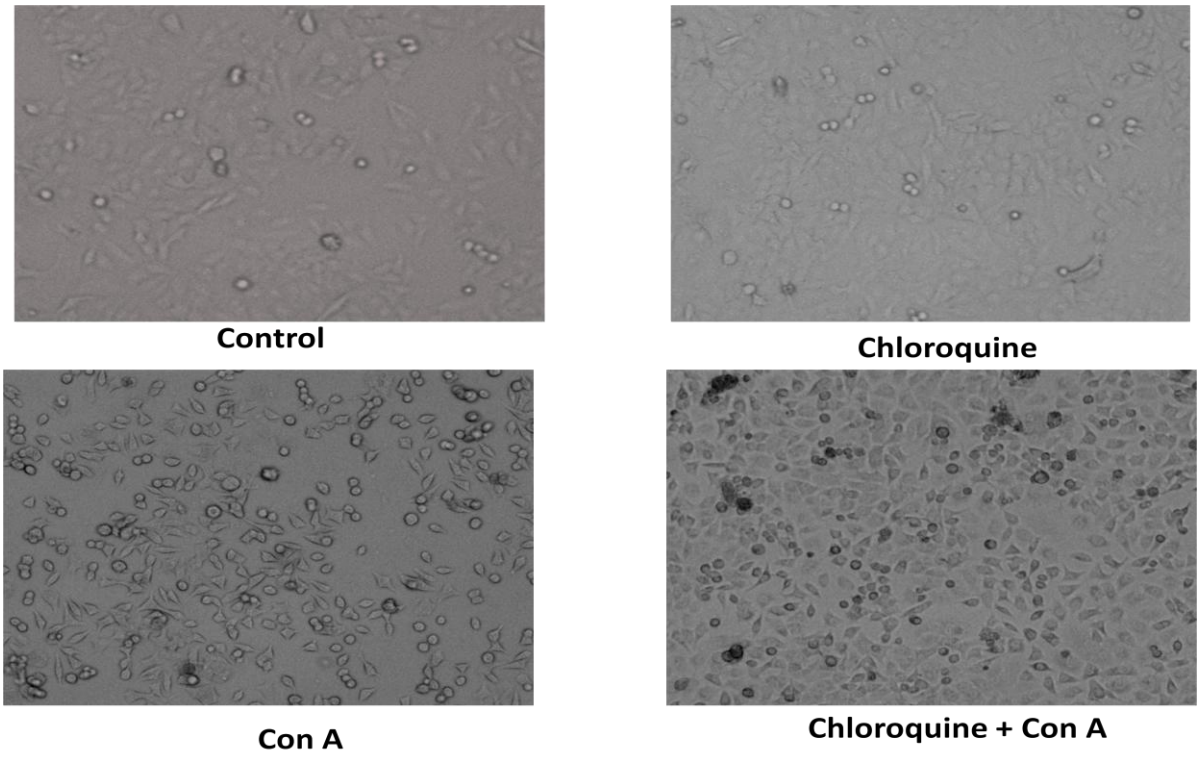

Fig-13: Observation of chloroquine (CQ) and Con A treatment in Hep-2 cell

INFERENCE

Chloroquine (CQ) is an autophagic inhibitor which inhibits autophagy. When CQ is added to the cells it was seen that there is no more cell death occurring and the cell death is same as the control. But when Con A is treated to the cells it was observed that highest number of cell death occurs. In last case when both CQ and Con A were treated to the cells, the number of dead cells decreses as compared to con A. Thus it indicates that CQ inhibits Con A induced cell death. (Fig-13)

CONCLUSION

The above study has shown that Con A plays a major role in both apoptosis and autophagy. From acridine orange staining it was confirmed that Con A induces autophagy in Hep-2 cells. Annexin V FITC staining, DAPI staining and Caspase-3/7 Glo Assay demonstrate that Con A triggered Apoptosis in Hep-2 cells. Above study also shown that CQ inhibits Con A induced cell death. Hence, from the above discussion it was conclude that Con A-induces autophagy followed to apoptosis in oral cancer. Therefore, open up a new perspective for Con A as potential anti-cancer drug for future Oral cancer therapeutics.

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